Detecting MUC-1 mRNA for diagnosing peripheral blood micro-metastasis in non-small cell lung cancer patients

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Background and Objective: Mucins, including MUC-1, are generally considered to be products of epithelial tissues and epithelial tumors. Theoretically, MUC-1 mRNA in peripheral blood with interstitial origin indicates metastasis. This study explores the feasibility of MUC-1 mRNA as a molecular marker in detecting peripheral blood micro-metastasis in patients with non-small cell lung cancer (NSCLC). Methods: Nested reverse transcription-polymerase chain reaction (RT-PCR) was used to detect MUC-1 gene expression in peripheral blood samples from 60 patients with NSCLC, 15 patients with benign pulmonary disease, and 20 healthy subjects. MUC-1 gene expression in K562 and HL60 cells with non-epithelial origin, and A549 and MCF-7 cells with epithelial origin were also detected by nested RT-PCR. Results: The positive rates of MUC-1 mRNA were 80.0% in the NSCLC group, 60.0% in the benign pulmonary disease group, and 65.0% in the healthy group. MUC-1 mRNA was also detected in K562, HL60, A549 and MCF-7 cells. Conclusion: MUC-1 mRNA can not be a reliable detection marker for peripheral blood micro-metastasis in NSCLC patients.

The incidence and mortality rate of lung cancer ranks number one among all malignant tumors. While the incidence in China is gradually increasing yearly, it is particularly higher in XuanWei and GeJiu area of YunNan province than in the rest of the nation. Unfortunately, long-term treatment outcome of lung cancer is still unsatisfactory. Study shows that local micro-metastasis is not easily detected by routine clinical examination is an important cause for tumor recurrence and metastasis. Current domestic and overseas investigations largely focus on the selection for molecular markers of micro-metastasis, mainly because of the lack of specific markers. This study used nested RT-PCR technology with sensitivity up to 10^6 to explore the possibility for MUC-1 gene as the micro-metastases marker in peripheral blood of non-small cell lung cancer patients.

Materials and Methods

Specimens. All 60 specimens were taken from patients hospitalized for surgery in the Thoracic Surgery Department of Yunnan Province Cancer Hospital from April 2007 to February 2008; patients had no radiotherapy and chemotherapy histories before admission and signed consent for use of blood samples. Among all patients, there were 12 cases of stage I cancer, 22 cases of stage II and 26 cases of stage III; 36 cases of adenocarcinoma and 24 cases of squamous cell carcinoma; 15 cases of benign lung diseases (tuberculosis, inflammatory pseudotumor and bulla) and 20 cases of healthy volunteers. Cell lines used included A549, MCF-7, K562 and HL60 from YunNan Institute of Oncology.

Reagents and primers design. Trizol used for RNA extraction was purchased from Invitrogen, the cDNA synthesis kit and DNA enzyme (DNase I, RNase-free) were purchased from Fermentas and the PCR reaction system was purchased from TIANGEN Biotech. We designed three pairs of primers for the MUC-1 gene using PrimerPremier 5.0 design software in reference to the Genbank sequence of MUC-1 (GeneID: 4582), respectively at 5' region, 3' region and cross the variable number of tandem repeat (VNTR) region of MUC-1. The primers sequences were:

(1) 5' region: A (sense) CCT CCC CAC CCA TTT CAC CA; B (antisense) AAC CTG AAG CTG GTT CCG TG.
(2) 3' region: C (sense) GGT ACC TCC TCT CAC CTC CTC CAA; D (antisense) CGT GGT GGA CAT TGA TGG TG.
(3) cross-VNTR region: E (sense) GTT CTT GTC TCT CAC CTC CTC CAA; F (antisense) CGT GGT GGA CAT TGA TGG TAC C.

At first, we used the above three pairs of primers to perform regular PCR amplification on the epithelium-originated cell lines: A549 and MCF-7 (conditions: 94°C 2 min, 94°C 45 s, 55°C 45 s, 72°C 1 min, 35 cycles and 72°C 7 min). PCR results showed that the 5' region primers yielded two bands of 300 and 330 bp, cross-VNTR primers yielded two bands of 280 and 330 bp...
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while the 3’ region primers only yielded a single band of 290 bp, therefore we chose the 3’ region primers as the inner primers for nested PCR. The sequences of the outer primers were: (sense) AGG CAA GTA GCA CTC ACC ATA G; (antisense) CAG CCA AGG CAA TGA GAT AGA C; with a amplified product of 510 bp. β-actin was included as an internal control and its primer sequences were: (sense) AGG TGG ATT CCG CTC CGG GCA; (antisense) ATC TTC CTG TCC CTC GAG CA; with an amplified product of 154 bp.

**Specimen collection and RNA extraction.** Four ml fresh blood was drawn from patients and the first 2 ml was discarded to avoid contamination of epithelial cells. The remaining 2 ml of blood was placed into an EDTA anticoagulant tube, lyzed red blood cell by red blood cell lysis buffer, separated nucleated cells and extracted total RNA using Trizol reagent (Invitrogen) by the Guanidine isothiocyanate/phenol extraction method. After total RNA extraction, we: (1) used DNase I (RNase-free) to digest the possible genomic DNA residue in order to prevent false positives during the PCR process; (2) used UV spectrophotometer to measure OD values to determine RNA purity and concentration; (3) used agarose gel electrophoresis to observe the RNA integrity.

**cDNA synthesis and nested PCR amplification.** Eligible RNA was used to synthesize the first chain of cDNA according to the instruction of the FermentascDNA synthesis kit. Amplification condition of the nested-PCR was as follows: first round: 94°C 5 min to inactivate reverse transcriptase and predenature, followed by 35 cycles of 94°C 45 s, 55°C 45 s and 68°C 1 min; final extension of 68°C 7 min. Second round: prenatdenatured at 94°C 2 min, 30 cycles of 94°C 45 s, 55°C 45 s and 72°C 1 min; final extension of 72°C 7 min. Amplification product was 287 bp.

**Sensitivity test.** When used lung cancer cell line A549, the sensitivity of nested RT-PCR amplification was up to 1 x 10⁶.

**Statistical analysis.** We used the software SPSS for windows 11.5 for statistical analysis, χ² test, and a Fisher exact test for difference detection. The criteria for statistical differences were p < 0.05 or p < 0.01.

**Results**

**Total RNA testing.** RNA extracted from all specimens had an OD260/280 value of 1.7–2.0 according to ultraviolet spectrophotometer. When separated by 1% agarose gel electrophoresis and stained by EB, the gel imaging system clearly detected the 28 s and 18 s bands, indicating the purity and integrity of RNA suitable for RT-PCR (Fig. 1).

**Nested-PCR amplification results.** The positive rate of MUC-1 mRNA expression in the peripheral blood of 60 cases of non-small cell lung cancer patients was 80.0% (48/60), as the 287 bp positive amplification band was detected in 48 cases. The positive expression of the target gene was detected in the peripheral blood of nine out of 15 cases of benign lung disease patients (60.0%) and in 13 out of 20 cases of healthy controls (65.0%). No significant difference in peripheral blood MUC-1 mRNA expression level was found between the patient group and the control group (p > 0.05), see Table 1. In addition, nested RT-PCR yielded the target gene amplification band in both non-epithelium originated (K562 and HL60) and epithelium-originated (A549 and MCF-7) cell lines. Electrophoresis results were shown in Figures 2–4.

**Discussion**

MUC-1 mucin is a highly glycolated protein with high molecular weight. Theoretically, it presents on the surface of normal gland cells and a variety of cancer cells. In general, human mucin genes have three common characteristics: (1) they consist of a variable number of tandem repeats (VNTRs); (2) their amino acid sequence encoded by VNTA enriches in serine and threonine; (3) their expression of RNA complex. The MUC1 gene locates at 1q21-24 and one important feature is its polymorphism, namely VNTRs in different individuals range from 20–120. Presently, most studies regarding MUC-1 examine its role in anti-tumor immunology as MUC1 is a trans-membrane glycoprotein, consisting of a peripheral scaffold plus carbohydrate chains; its peptide skeleton contains continuously repetitive sequences with stable and consistent spatial structures to form many identical, repeated epitopes. These epitopes can be recognized by antibodies, and they multivalently bind to T cell receptors as super-antigens to activate T cells. However, these peptide epitopes are covered by peripheral carbohydrate chains on MUC1 when expressed normally, and therefore cannot be recognized. In contrast, at the cancer cell surface, these epitopes are exposed because of the incomplete MUC1 glycosylation and loss of polarity, etc., and they may become the target for tumor-specific immunotherapy.
In recent years, since MUC-1 is regarded as the product of epithelial tissue and the tumor originates from the epithelium, detection of MUC-1 mRNA or its product in mesenchyma-origi- 
nated blood suggests cancer metastasis. Many domestic and foreign scholars consider this gene as a molecular marker for the detection of tumor micro-metastases, and many investigators in China also report that MUC-1 mRNA is a good indicator for evaluating the peripheral blood micro-metastases of epithelium-originated tumors. However, in this study, we found a high level of target gene expression not only in the peripheral blood of patients with lung cancer, but also in the peripheral blood of benign disease controls and healthy subjects, as well as in non-epithelium originated cell lines K562 and HL60. Therefore, after ruling out possible causes of false-positive such as: contamination during procedures, interference by genomic DNA, improper primer design etc., one remaining possibility is that the nuclear cells in peripheral blood cells may have a low level of intrinsic MUC-1 expression, which can be detected by a highly sensitive nested-RT-PCR technique, consistent with the results by Dent et al. who show the expression of MUC-1 not only in nuclear cells separated from peripheral blood, but also in cell lines originated from hematopoietic cells by RT-PCR and western blot methods; therefore they believe that MUC-1 is not suitable as the marker for micro-metastases of epithelial cell-originated tumors. Saintigny et al. use CK19, CK7 and MUC-1 mRNA as indexes to detect peripheral blood and lymphatic node micro-metastases in non-small cell lung cancer patients by a real-time quantitative RT-PCR method, proving that MUC-1 mRNA not only expresses at a high level in the patient group, but also in the peripheral blood of healthy volunteers. Zhong et al. report that the use of RT-PCR and immunohistochemistry methods to detect MUC-1 as the molecular marker for micrometastasis of peripheral blood and bone marrow cell-derived tumors is not specific. Our study showed no statistical difference between the control group and the lung cancer group through statistical analysis, indicating the low specificity of using MUC-1 mRNA as the marker to detect non-small cell lung cancer micro-metastases.

As early as 1997, Oossterkamp et al. applied RT-PCR, northern blot, and radioactive immunoprecipitation methods to explore the MUC-1 expression status in human epithelial and non-epithelial derived cells lines. They detected the presence of MUC-1 mRNA not only in epithelial tissue-originated cells, but also in astrocytoma, melanoma and neuroblastoma cells (etcetera) in spite of a much lower expression level of MUC-1 mRNA in non-epithelial tumor cells than in epithelial tumors. Other scholars have reported similar results, which indicate that MUC-1 may express at a low level in many tissues and cells other than epithelial tissue, rendering its low specificity as a marker for micro-metastasis. In this study, we used nested RT-PCR technology, which was 100-fold more sensitive than the common RT-PCR technology, and obtained consistent result with other investigators using common RT-PCR technology. Furthermore, since methods such as western blot, northern blot, real-time quantitative RT-PCR etc., are also used in their reports to obtain the same result, we
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believe that the possibility of whether MUC-1 mRNA can be used as a marker to detect non-small cell lung cancer peripheral blood micro-metastases still needs further exploration.

References


